VIROLOGY 244, 161–172 (1998)
ARTICLE NO. VY989092

Transcripts of a Chimeric cDNA Clone of Hepatitis C Virus Genotype 1b Are Infectious *in Vivo*

Masayuki Yanagi,* Marisa St. Claire,† Max Shapiro,† Suzanne U. Emerson,* Robert H. Purcell,* and Jens Bukh*,[1]

*Hepatitis Viruses Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland; and †Bioqual, Inc., Rockville, Maryland

Received December 30, 1997; returned to author for revision January 21, 1998; accepted February 11, 1998

We constructed a chimeric cDNA clone of hepatitis C virus (HCV) that is infectious. The chimeric genome encodes the polyprotein of a genotype 1b strain (HC-J4) of HCV and replicates via 5′ and 3′ untranslated regions of a genotype 1a strain. The infectivity of three full-length cDNA clones was tested by direct injection of RNA transcripts into the liver of a chimpanzee. The chimpanzee became infected with HCV and the viral titer increased over time from 10^2 genome equivalents (GE)/ml at week 1 postinoculation (p.i.) to 10^4–10^5 GE/ml during weeks 3–11 p.i. Antibodies to HCV were detected from week 18 p.i. However, the chimpanzee did not develop hepatitis. Sequence analysis of PCR products amplified from the serum of the chimpanzee demonstrated that only one of the three clones was infectious. Sequence comparisons with the cloning source, an acute-phase infectious plasma pool derived from an experimentally infected chimpanzee, showed that this infectious clone had three amino acids that differed from the consensus sequence of HC-J4, whereas the two noninfectious clones had seven and nine amino acid differences, respectively. Together, genotype 1b, represented by the infectious molecular clone described herein, and genotype 1a, represented by the two cDNA clones previously shown to be infectious for chimpanzees, account for the majority of HCV infections in the United States, Europe, and Japan. © 1998 Academic Press

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of chronic liver disease (Houghton, 1996). More than 80% of individuals infected with HCV become chronically infected, with about 4 million people infected in the United States (Alter, 1997; Hoofnagle, 1997). Chronically infected individuals have a relatively high risk of developing chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Hoofnagle, 1997). The only effective therapy for chronic hepatitis C, interferon (IFN), induces a sustained response in less than 25% of treated patients (Fried and Hoofnagle, 1995). Consequently, HCV is currently the most common cause of end-stage liver failure and the reason for about 30% of liver transplants performed in the United States (Hoofnagle, 1997). There is no vaccine for HCV. Although the number of acute HCV infections has declined, in part because of effective screening of blood and blood products, it is estimated that there are still more than 25,000 new infections yearly in the United States (Alter, 1997). Thus, HCV constitutes a serious public health problem.

Hepatitis C virus has a positive-sense single-strand RNA genome and is a member of the virus family Flaviviridae (Choo *et al.*, 1991; Rice, 1996). The viral genome of approximately 9600 nucleotides (nt) consists of a highly conserved 5′ untranslated region (UTR), a single long open reading frame (ORF) of approximately 9000 nt, and a complex 3′ UTR. The 5′ UTR contains an internal ribosomal entry site (Tsukiyama-Kohara *et al.*, 1992; Honda *et al.*, 1996). The 3′ UTR consists of a short variable region, a polypyrimidine tract of variable length, and, at the 3′ end, a highly conserved region of approximately 100 nt (Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1995, 1996; Yamada *et al.*, 1996). The last 46 nucleotides of this conserved region were predicted to form a stable stem-loop structure thought to be critical for viral replication (Blight and Rice, 1997; Ito and Lai, 1997; Tsuchihara *et al.*, 1997). The ORF encodes a large polyprotein precursor that is cleaved into at least 10 proteins by host and viral proteinases (Rice, 1996). The predicted envelope proteins contain several conserved N-linked glycosylation sites and cysteine residues (Okamoto *et al.*, 1992a). The NS3 gene encodes a serine proteinase and an RNA helicase and the NS5B gene encodes an RNA-dependent RNA polymerase.

Globally, six major HCV genotypes (genotypes 1–6) and multiple subtypes (a, b, c, etc.) have been identified (Bukh *et al.*, 1993; Simmonds *et al.*, 1993). The most divergent HCV isolates differ from each other by more than 30% over the entire genome (Okamoto *et al.*, 1992a). Infection with genotype 1 is most prevalent (Bukh *et al.*, 1995). In the United States, HCV genotypes 1a and 1b constitute the majority of infections. In many other areas,

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. AF054247–AF054268.

[1] To whom correspondence and reprint requests should be addressed at NIH, NIAID, LID, Hepatitis Viruses Section, Building 7, Room 201, 7 Center Drive, MSC 0740, Bethesda, MD 20892-0740. Fax: (301) 402-0524.

0042-6822/98 $25.00
Copyright © 1998 by Academic Press
All rights of reproduction in any form reserved.

especially in Europe and Japan, genotype 1b predominates. Recently, a number of studies have suggested that the severity of liver disease and the outcome of therapy may be genotype-dependent (reviewed in Bukh *et al.*, 1997). In these studies infection with HCV genotype 1b was associated with more severe liver disease (Brechot, 1997) and a poorer response to IFN therapy (Fried and Hoofnagle, 1995). However, the exact biological consequences of the genetic heterogeneity of HCV are still unclear.

HCV circulates in an infected individual as a quasispecies of closely related genomes (Bukh *et al.*, 1995; Farci *et al.*, 1997). The genetic heterogeneity of HCV is not distributed uniformly throughout the genome but is concentrated in hypervariable region 1 (HVR1) at the amino-terminal end of E2 (Weiner *et al.*, 1991; Hijikata *et al.*, 1991). The HVR1 region contains a neutralization epitope (Farci *et al.*, 1996; Shimizu *et al.*, 1996) and the amino acid sequence of HVR1 can undergo sequential changes during infection, probably resulting in escape from immune surveillance by the host and establishment of persistent HCV infection (Weiner *et al.*, 1992; Farci *et al.*, 1997). A second hypervariable region was identified in E2 (HVR2) but only in genotype 1b strains of HCV (Hijikata *et al.*, 1991). The quasispecies nature of HCV might also impact the severity of associated liver disease and resistance to IFN treatment (reviewed in Farci *et al.*, 1997).

Hepatitis C virus infects some continuous human T cell lines *in vitro* (Shimizu *et al.*, 1992) but replicates poorly in such cell cultures. The chimpanzee is the only nonhuman host of HCV (Farci *et al.*, 1993), but its availability is very limited. This lack of a convenient animal model or an efficient *in vitro* propagation system has made it difficult to study the virological characteristics of HCV or to develop antiviral therapies and vaccines.

Recently, two research groups independently constructed an infectious cDNA clone of strain H77 (genotype 1a) of HCV (Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997). In both studies, RNA transcripts encoding the consensus amino acid sequence of the putative polyprotein were infectious for chimpanzees, whereas those encoding a nonconsensus polyprotein were not. These infectious clones should aid in studying HCV replication and pathogenesis and should provide an important tool for development of *in vitro* replication and propagation systems. However, given the extensive genetic heterogeneity of HCV, it will probably be important to have infectious clones of more than one genotype. Although the near-complete genomic sequences of several HCV strains have been published, past experience suggests that it will be difficult to clone the infectious sequence. First, the viral quasispecies possibly include a proportion of defective or noninfectious genomes. Second, clinical samples obtained from patients or experimentally infected chimpanzees contain only minute amounts of viral RNA. Therefore, it is necessary to perform *in vitro* amplification of viral RNA by reverse transcription (RT) and PCR, which can introduce errors into the cDNA derived from the viral genomes.

In the present study, we performed a detailed sequence analysis of the HC-J4 strain of genotype 1b that had been biologically amplified in a chimpanzee and constructed an infectious chimeric cDNA clone by inserting the complete ORF of the genotype 1b strain into a cassette vector that incorporated part of the untranslated regions of an infectious clone of a genotype 1a strain.

RESULTS

Quasispecies of strain HC-J4 in the infectious plasma pool used as the cloning source

An infectious cDNA clone of a genotype 1a strain of HCV had been obtained only after the ORF was engineered to encode the consensus polyprotein (Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997). Thus, prior to constructing an infectious cDNA clone of a 1b genotype, we performed a detailed sequence analysis of the cloning source to determine the consensus sequence. A plasma pool of strain HC-J4 was prepared from acute-phase plasmapheresis units collected from a chimpanzee experimentally infected with HC-J4/91 (Okamoto *et al.*, 1992b). This HCV pool had a PCR titer of 10^4–10^5 GE/ml and an infectivity titer of approximately 10^3 chimpanzee infectious doses/ml (Bukh *et al.*, unpublished data).

We determined the heterogeneity of the 3′ UTR of strain HC-J4 by analyzing 24 clones of nested RT–PCR product. The consensus sequence was identical to that previously published for HC-J4/91 (Okamoto *et al.*, 1992b), except at position 9407 (see below). The variable region consisted of 41 nucleotides (nt 9372–9412), including two in-frame termination codons. Furthermore, its sequence was highly conserved except at positions 9399 (19 A and 5 T clones) and 9407 (17 T and 7 A clones). The poly(U-UC) region varied slightly in composition and greatly in length (31–162 nucleotides). In the conserved region, the first 16 nucleotides of 22 clones were identical to those previously published for other genotype 1 strains, whereas 2 clones each had a single point mutation. These data suggested that the structural organization at the 3′ end of HC-J4 was similar to that of our infectious clone of a genotype 1a strain.

We next amplified the entire ORF of HC-J4 in a single round of long RT-PCR (Fig. 1). Our original plan was to clone the resulting PCR products into the *Pin*AI and *Bri*I site of a HCV cassette vector (pCV), which had fixed 5′ and 3′ termini of genotype 1a (Yanagi *et al.*, 1997). However, we were not able to obtain full-length clones. There-

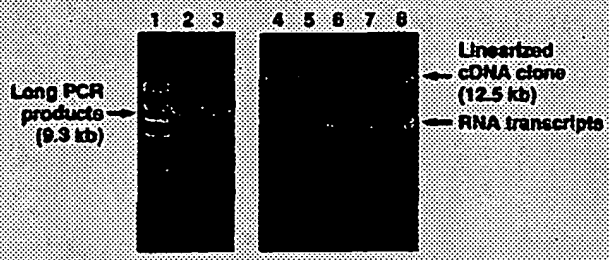

FIG. 1. Agarose gel of long RT–PCR amplicons and transcription mixtures. Lanes 1 and 4: Molecular weight marker (λ/*Hin*dIII digest). Lanes 2 and 3: RT–PCR amplicons of the entire ORF of HC-J4. Lane 5: pCV-H77C transcription control (Yanagi *et al.*, 1997). Lanes 6–8: 1/40 of each transcription mixture of pCV-J4L2S, pCV-J4L4S, and pCV-J4L6S, respectively, which was injected into the chimpanzee.

fore, we separately subcloned into pCV two genome fragments (L and S) derived from the long RT–PCR products (Fig. 2).

To determine the consensus sequence of the ORF we sequenced nine clones each of the L fragment (pCV-J4L) and of the S fragment (pCV-J4S). We found a quasispecies at 275 nucleotide (3.05%) and 78 amino acid (2.59%) positions, scattered throughout the 9030 nt (3010 aa) of the ORF (Fig. 3). Of the 161 nucleotide substitutions unique to a single clone, 71% were at the third position of the codon and 72% were silent.

Each of the nine L clones represented the near-complete ORF of an individual genome. The differences among the L clones were 0.30–1.53% at the nucleotide and 0.31–1.47% at the amino acid level (Fig. 4). Two clones, L1 and L7, had a defective ORF due to a single nucleotide deletion and a single nucleotide insertion, respectively. Even though the HC-J4 plasma pool was obtained in the early acute phase, it appeared to contain at least three viral species (Fig. 5). Species A contained the L1, L2, L6, L8, and L9 clones, species B the L3, L7, and L10 clones, and species C the L4 clone. Although each species A clone was unique, all A clones differed from all B clones at the same 20 amino acid sites (Fig. 3). At these positions, species C had the species A sequence at 14 positions and the species B sequence at 6 positions (Fig. 3).

Okamoto and co-workers (Okamoto *et al.*, 1992b) previously determined the nearly complete genome consensus sequence of strain HC-J4 in acute-phase serum of the first chimpanzee passage (HC-J4/83) as well as in chronic-phase serum collected 8.2 years later (HC-J4/91). In addition, they determined the sequence of amino acids 379 to 413 (including HVR1) and amino acids 468 to 486 (including HVR2) of multiple individual clones (Okamoto *et al.*, 1992b). We found that the sequences of individual genomes in the plasma pool collected from a chimpanzee inoculated with HC-J4/91 were all more closely related to HC-J4/91 than to HC-J4/83 (Figs. 4 and 5) and contained HVR amino acid sequences closely related to three of the four viral species previously found in HC-J4/91 (Fig. 6).

The difficulty of determining the consensus sequence of HC-J4 in the plasma pool

We determined the consensus sequence of nucleotides 156–9371 of HC-J4 by two approaches. In one approach, the consensus sequence was deduced from nine clones of the long RT–PCR product. In the other approach the long RT–PCR product was reamplified by PCR as overlapping fragments which were sequenced directly. The two "consensus" sequences differed at 31 (0.34%) of 9216 nucleotide positions and at 11 (0.37%) of 3010 deduced amino acid positions (Fig. 3). At all of these positions a major quasispecies of strain HC-J4 was found in the plasma pool. At 9 additional amino acid positions the cloned sequences displayed heterogeneity and the direct sequence was ambiguous (Fig. 3). Finally, it should be noted that there were multiple amino acid positions at which the consensus sequence obtained by direct sequencing was identical to that obtained by cloning and sequencing even though a major quaisispecies was detected (Fig. 3).

For positions at which the two "consensus" sequences of HC-J4 differed, we included both amino acids in a composite consensus sequence (Fig. 3). However, even with this allowance, none of the nine L clones analyzed (aa 1–2864) had the composite consensus sequence: two clones did not encode the complete polyprotein and the remaining seven clones differed from the consensus sequence by 3–13 amino acids (Fig. 3).

Chimeric full-length cDNA clones containing the entire ORF of HC-J4

Three full-length cDNA clones were constructed by cloning different L fragments into the *Pin*AI/*Bgl*II site of pCV-J4S9, the cassette vector for genotype 1a (Fig. 2), which also contained an S fragment encoding the consensus amino acid sequence of HC-J4. Therefore, although the ORF was from strain HC-J4, most of the 5′ and 3′ terminal sequence originated from strain H77 (Yanagi *et al.*, 1997). As a result, the 5′ and 3′ UTR were chimeras of genotypes 1a and 1b (Fig. 7). The first 155 nucleotides of the 5′ UTR were from strain H77 (genotype 1a) and differed from the authentic sequence of HC-J4 (genotype 1b) at nucleotides 11, 12, 13, 34, and 35. In two clones (pCV-J4L2S, pCV-J4L6S) the rest of the 5′ UTR had the consensus sequence of HC-J4, whereas the third clone (pCV-J4L4S) had a single nucleotide insertion at position 207. In all three clones the first 27 nucleotides of the 3′ variable region of the 3′ UTR were identical with the consensus sequence of HC-J4. The remaining 15 nucleotides of the variable region, the poly(U-UC) region, and the 3′ conserved region of the 3′ UTR had the same

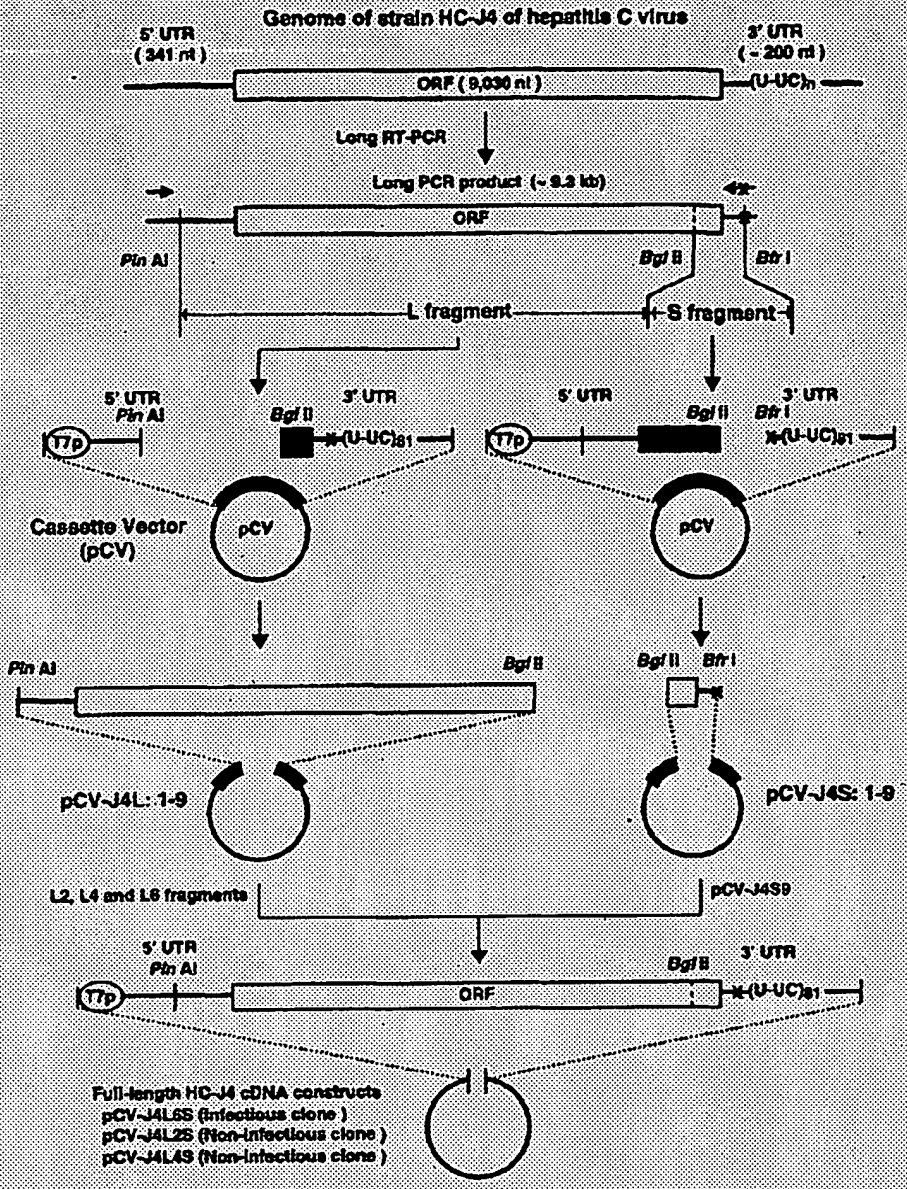

FIG. 2. Strategy for the construction of full-length cDNA clones of HCV strain HC-J4. The long PCR products were cloned as two separate fragments (L and S) into a cassette vector (pCV) with fixed 5' and 3' termini of HCV (Yanagi *et al.*, 1997). Full-length cDNA clones of HC-J4 were obtained by inserting the L fragment from three pCV-J4L clones into three identical pCV-J4S9 clones after digestion with *Pin*AI (isoschizomer of *Age*I) and *Bgl*II.

sequence as an infectious clone of strain H77 (Yanagi *et al.*, 1997).

None of the three full-length clones of HC-J4 had the ORF composite consensus sequence (Figs. 3 and 8). The pCV-J4L6S clone had only three amino acid changes: Q for R at position 231 (E1), V for A at position 937 (NS2), and T for S at position 1215 (NS3). The pCV-J4L4S clone had seven amino acid changes, including a change at position 450 (E2) that eliminated a highly conserved N-linked glycosylation site (Okamoto *et al.*, 1992a). Finally, the pCV-J4L2S clone had nine amino acid changes compared with the consensus sequence of HC-J4. A change at position 304 (E1) mutated a highly conserved cysteine residue (Bukh *et al.*, 1993; Okamoto *et al.*, 1992a).

Transfection of a chimpanzee by transcripts from a chimeric cDNA clone

We tested the infectivity of RNA transcripts from the three chimeric cDNA clones simultaneously in a chimpanzee. The chimpanzee became infected with HCV as measured by increasing titers of 10^2 GE/ml at week 1 p.i., 10^3 GE/ml at week 2 p.i., and 10^4–10^5 GE/ml during weeks 3 to 11 p.i. (Fig. 9). The chimpanzee was negative for HCV RNA at weeks 19 and 20 p.i. (see Note added in

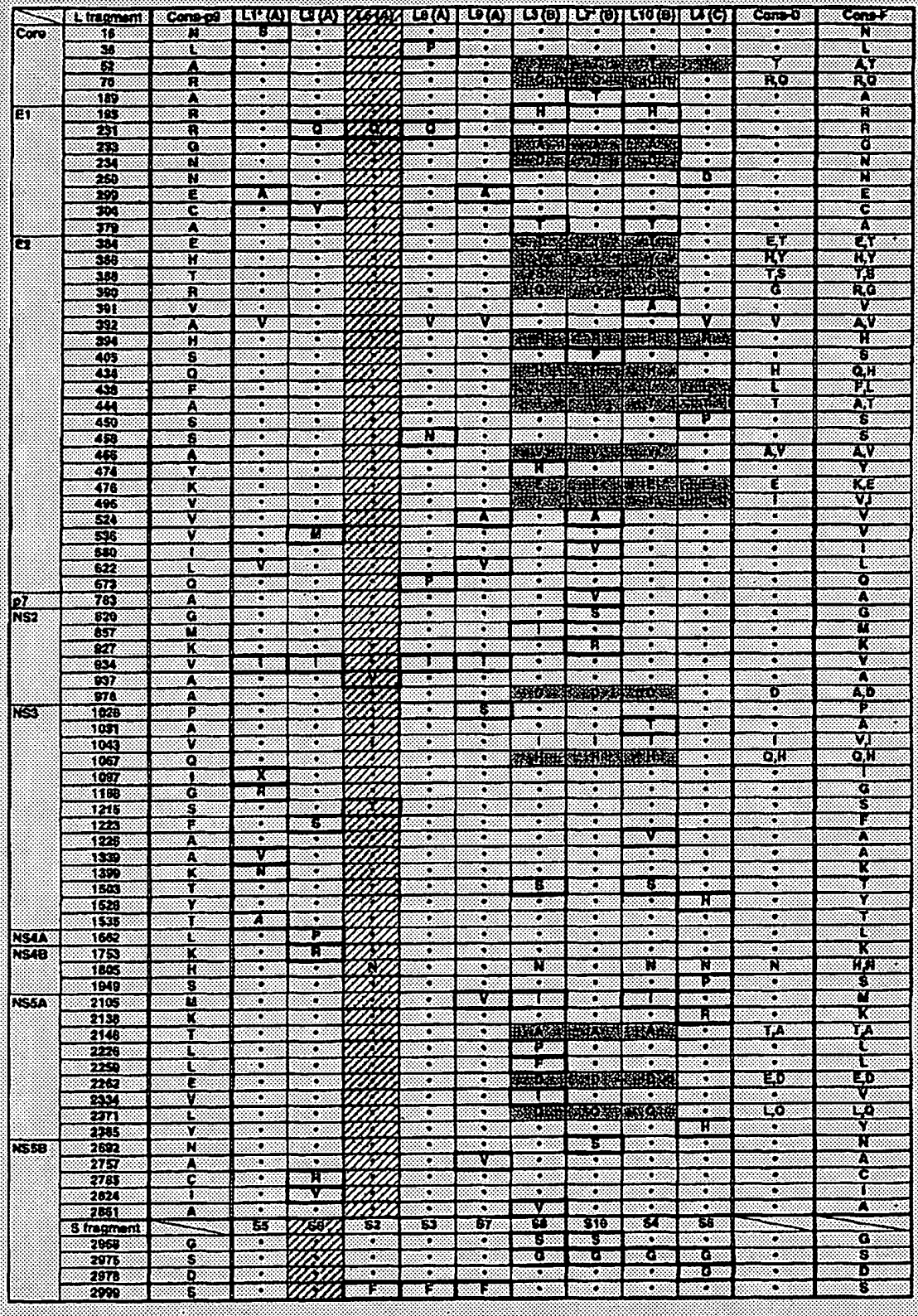

FIG. 3. Amino acid positions with a quasispecies of HC-J4 in the acute-phase plasma pool obtained from an experimentally infected chimpanzee. Cons-p9: consensus amino acid sequence deduced from analysis of nine L fragments and nine S fragments (see Fig. 2). Cons-D: consensus sequence derived from direct sequencing of the PCR product. A–C: groups of similar viral species. Dot: amino acid identical to that in Cons-p9. Capital letter: amino acid different from that in Cons-p9. Cons-F: composite consensus amino acid sequence combining Cons-p9 and Cons-D. Boxed amino acid: different from that in Cons-F. Shaded amino acid: different from that in all species A sequences. An asterisk indicates defective ORF due to a nucleotide deletion (clone L1, aa 1097) or insertion (clone L7, aa 2770). Diagonal lines: fragments used to construct the infectious clone.

nt / aa	L1 (A)	L2 (A)	L6 (A)	L8 (A)	L9 (A)	L3 (B)	L7(B)	L10 (B)	L4 (C)	HC-J4/91	HC-J4/83
L1 (A)		0.26	0.30	0.42	0.50	1.50	1.53	1.46	0.95	0.83	1.79
L2 (A)	0.50		0.45	0.53	0.57	1.49	1.51	1.45	0.98	0.82	1.77
L6 (A)	0.52	0.42		0.30	0.35	1.33	1.38	1.29	0.80	0.68	1.58
L8 (A)	0.42	0.38	0.31		0.36	1.32	1.34	1.28	0.79	0.65	1.62
L9 (A)	0.55	0.52	0.45	0.35		1.42	1.42	1.38	0.91	0.75	1.66
L3 (B)	1.47	1.43	1.15	1.33	1.36		0.61	0.30	1.43	0.90	1.51
L7 (B)	1.36	1.33	1.12	1.22	1.22	0.66		0.57	1.47	0.95	1.54
L10 (B)	1.36	1.33	1.05	1.22	1.26	0.31	0.56		1.37	0.85	1.42
L4 (C)	0.77	0.80	0.59	0.63	0.70	1.12	1.08	1.01		0.76	1.73
HC-J4/91	0.94	0.91	0.63	0.80	0.87	0.77	0.73	0.66	0.52		1.22
HC-J4/83	1.96	1.89	1.68	1.85	1.82	1.75	1.61	1.57	1.71	1.40	

FIG. 4. Comparisons (percent difference) of nucleotide (nt 156–8935) and predicted amino acid sequences (aa 1–2864) of L clones (species A–C, this study), HC-J4/91 (Okamoto *et al.*, 1992b), and HC-J4/83 (Okamoto *et al.*, 1992b). Differences among species A sequences and among species B sequences are shaded.

proof) and antibodies to HCV were detected (weeks 18–20 p.i.). Serum liver enzyme levels were within normal range throughout follow-up.

To identify which of the three full-length HC-J4 clones were infectious, we cloned and sequenced the NS3 region (nt 3659–4110) of HCV genomes amplified by RT–PCR from serum samples taken from the infected chimpanzee during weeks 2 and 4 p.i. The PCR primers were a complete match with each of the original three clones. Thus, this assay should not have preferentially amplified one virus over another. Sequence analysis of 26 and 24 clones obtained at weeks 2 and 4 p.i., respectively, demonstrated that all originated from the transcripts of pCV-J4L6S. The consensus sequence of PCR products of the nearly complete genome (nt 11–9441), amplified from serum obtained during week 2 p.i., was identical to the sequence of pCV-J4L6S and there was no evidence of quasispecies. Thus, RNA transcripts of pCV-J4L6S, but not of pCV-J4L2S or pCV-J4L4S, were infectious *in vivo*.

The chimeric sequences of genotypes 1a and 1b in the UTRs were maintained in the infected chimpanzee. The consensus sequence of nucleotides 11–341 of the 5′ UTR and the variable region of the 3′ UTR, amplified from serum obtained during weeks 2 and 4 p.i., had the expected chimeric sequence of genotypes 1a and 1b (Fig. 7). Also, three of four clones of the 3′ UTR obtained at week 2 p.i. had the chimeric sequence of the variable

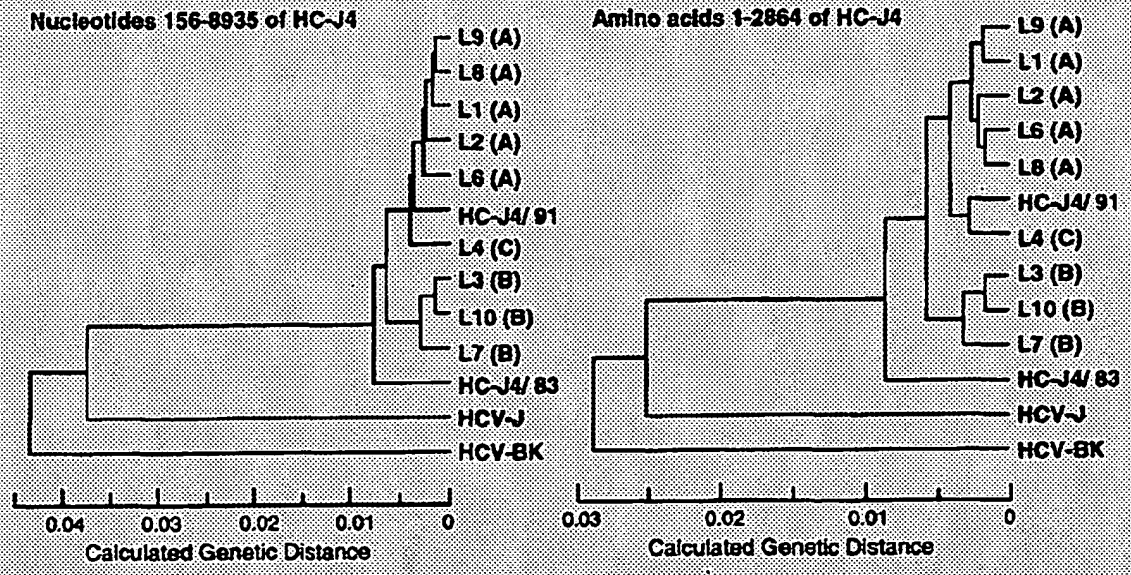

FIG. 5. UPGMA trees of HC-J4/91 (Okamoto *et al.*, 1992b), HC-J4/83 (Okamoto *et al.*, 1992b), two prototype strains of genotype 1b (HCV-J, Kato *et al.*, 1990; HCV-BK, Takamizawa *et al.*, 1991), and L clones (this study).

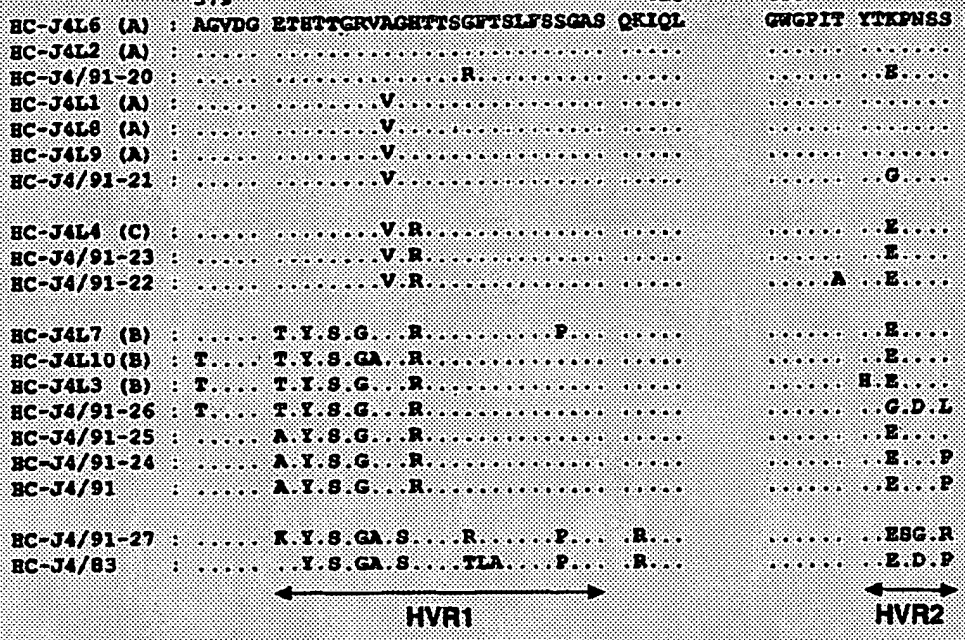

FIG. 6. Alignment of the HVR1 and HVR2 amino acid sequences of the E2 of HCV. The sequences of nine L clones of HC-J4 (species A–C) obtained from an early acute-phase plasma pool of an experimentally infected chimpanzee were compared with the sequences of eight clones (HC-J4/91-20 through HC-J4/91-27, Okamoto *et al.*, 1992b) derived from the inoculum. Dot: an amino acid identical to that in the top line. Capital letters: amino acid different from that in the top line.

region, whereas a single substitution was noted in the fourth clone. However, in all four clones the poly(U) region was longer (2–12 nt) than expected. Also, we observed extra C and G residues in this region. For the most part, the number of C residues in the poly(UC) region was maintained in all clones, although the spacing varied. As we showed previously, variations in the number of U residues can reflect artifacts introduced during PCR amplification (Yanagi *et al.*, 1997). The sequence of the first 19 nucleotides of the conserved region was maintained in all four clones. Thus, with the exception of the poly(U-UC) region, the genomic sequences recovered from the infected chimpanzee were exactly those of the chimeric infectious clone.

DISCUSSION

After much effort, the first cDNA clones of HCV that are infectious for chimpanzees were recently constructed (Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997). Both of these previously reported clones were derived from strain H77, which is genotype 1a. We used the cassette vector we developed to clone strain H77 to construct an infectious cDNA clone containing the ORF of a second subtype. This new clone contains a chimeric HCV genome, which is composed mostly of genotype 1b sequences from strain HC-J4. The encoded polyproteins of genotypes 1a and 1b share only about 85% identity. Genotype 1b is the most prevalent genotype of HCV in the United States, Europe, and Japan. The availability of infectious clones representing two important subtypes of genotype 1 should provide new ways of studying this virus.

There is no effective *in vitro* propagation or replication system for HCV. Thus, the infectivity of HCV clones has been determined by *in vivo* transfection: viral nucleic acid is injected directly into the liver of a chimpanzee. The previous transfection protocols had required laparotomy (Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997), which can be performed only once per animal. In the present study, we demonstrated that the *in vivo* transfection could be performed by ultrasound-guided percutaneous intrahepatic injection (St. Claire *et al.*, unpublished data). This less invasive procedure should facilitate *in vivo* studies of cDNA clones of HCV in chimpanzees, since percutaneous procedures can be performed repeatedly.

We demonstrated that RNA transcripts of one cDNA clone of HC-J4, but not of two other clones with different ORFs but the same termini, were infectious *in vivo*. We injected the same amount of cDNA and transcription mixture for each of the clones (Fig. 1) so the failures to infect were not due to insufficient RNA. In the previous two reports on infection of chimpanzees only those clones engineered to have the independently determined and slightly different consensus amino acid sequence of the polyprotein of strain H77 were infectious (Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997). Although the two infectious clones differed at 4 amino acid positions, these differences were represented in a major component of the quasispecies of the cloning source (Yanagi *et*

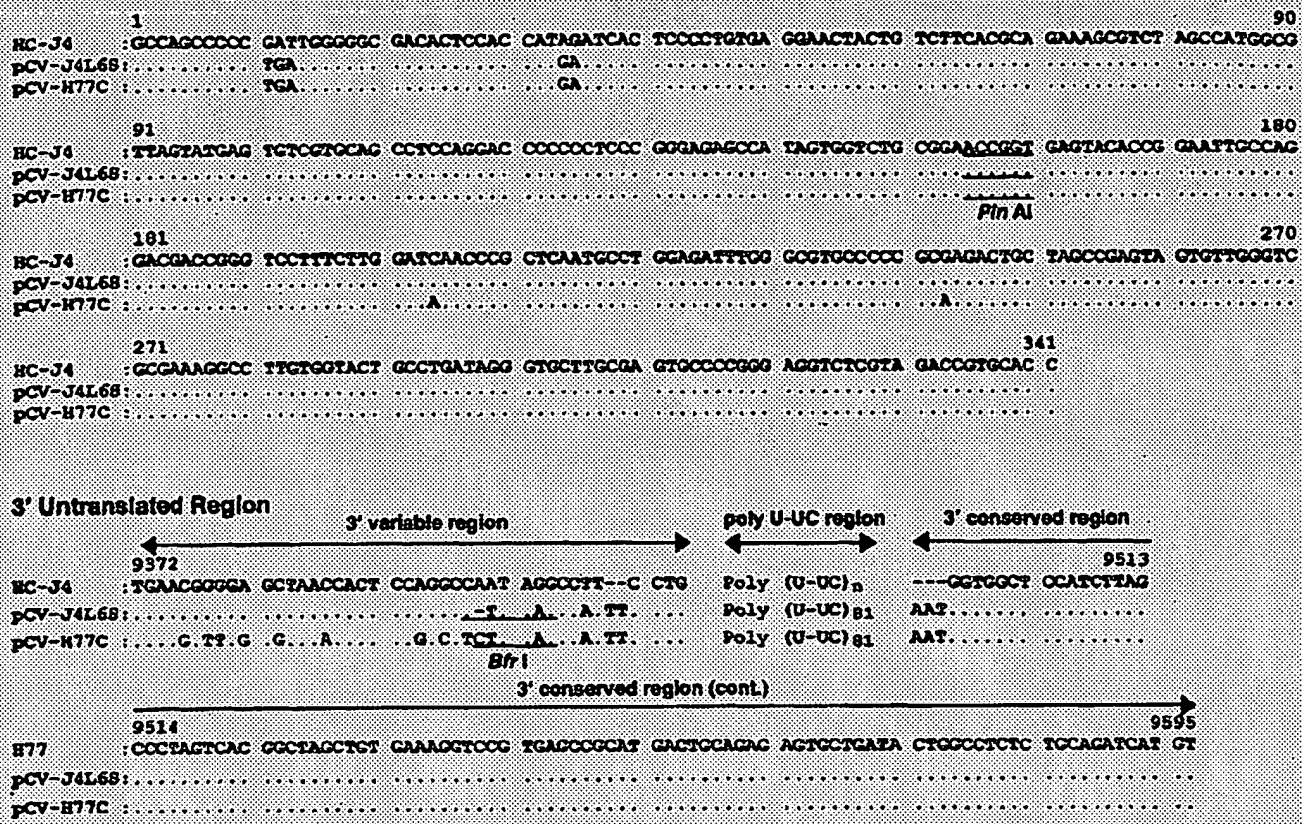

FIG. 7. Alignment of the 5' and the 3' UTR sequences of infectious clones of genotype 1a (pCV-H77C) and 1b (pCV-J4L6S). Top line: consensus sequence of the indicated strain. Dot: identity with consensus sequence. Capital letter: different from the consensus sequence. Dash: deletion. Underlined: *Pin*AI and *Bfr*I cleavage site. Numbering corresponds to the HCV sequence of pCV-J4L6S.

al., unpublished data) and therefore did not represent substantial differences. In the present study, we were unable to define a single consensus sequence of strain HC-J4, because the consensus sequence obtained by two different approaches (direct sequencing and sequencing of cloned products) differed at 20 amino acid positions, even though the same genomic PCR product was analyzed. The infectious clone differed at 2 positions from the composite amino acid consensus sequence, from the sequence of the eight additional HC-J4 clones analyzed in this study and from published sequences of earlier passage samples. An additional amino acid differed from the composite consensus sequence but was found in two other HC-J4 clones analyzed in this study. The two noninfectious full-length clones of HC-J4 differed from the composite consensus sequence by only 7 and

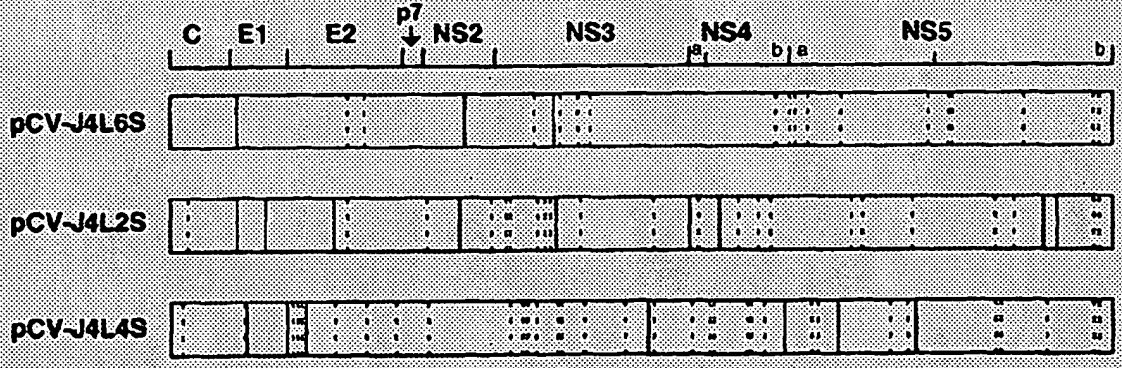

FIG. 8. Comparison of the ORF of individual full-length cDNA clones of HCV strain HC-J4 with the consensus sequence (see Fig. 3). Solid lines: amino acid changes. Dashed lines: silent mutations. Clone pCV-J4L6S was infectious *in vivo*, whereas clones pCV-J4L2S and pCV-J4L4S were not.

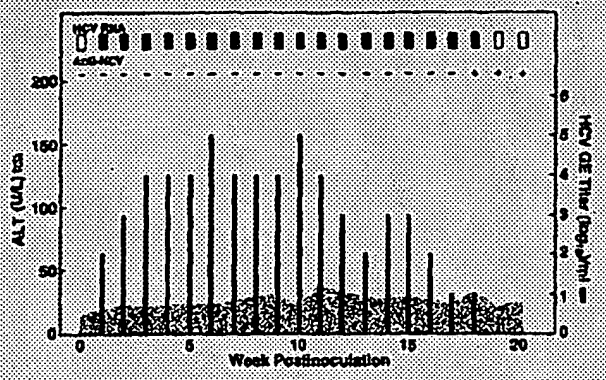

FIG. 9. Course of infection with hepatitis C virus (HCV) in chimpanzee 1500 following transfection with the infectious cDNA clone pCV-J4L6S (strain HC-J4, genotype 1b). Results of qualitative RT-nested PCR for HCV RNA (filled rectangles, positive; empty rectangles, negative) and second-generation ELISA test for anti-HCV ((+) positive; (−) negative) are shown. Serum levels of alanine aminotransferase (ALT; shaded area) and the $\log_{10}$ HCV genome equivalent titer (vertical columns) are plotted against time.

9 amino acid differences. However, since these clones had the same termini as the infectious clone (except for a single nucleotide insertion in the 5′ UTR of pCV-J4L4S), one or more of these amino acid changes in each clone was apparently deleterious for the virus. Although we have formally demonstrated for the first time that HCV polyprotein sequences other than the consensus sequence can be infectious, our data confirmed that deviations from the consensus sequence are often lethal.

The genome termini must interact with the viral polymerase during initiation of viral replication. Since the amino acid sequence of the putative RNA-dependent RNA polymerases of HC-J4 and H77 varied by more than 10% it was not obvious that the polymerase of HC-J4 would be able to replicate a chimeric genome containing portions of the H77 termini. The 5′ UTR sequence of the infectious genotype 1b clone differed from the sequence of the original genotype 1b virus at 5 nucleotide positions, all close to the 5′ end where they might be expected to affect RNA replication. In the 3′ UTR of this infectious clone, the 3′ variable sequence was chimeric and represented strain H77 rather than strain HC-J4 in 5 of 42 positions. Thus, although the 3′ variable sequence was highly conserved within HC-J4, this strain-specific sequence was not critical for replication by the HC-J4 polymerase. In the present study, we found that HC-J4, like other strains of genotype 1b (Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1996; Yamada *et al.*, 1996), had a poly(U-UC) region followed by a terminal conserved element. The poly(U-UC) region appears to vary considerably, so it was not clear whether changes in this region would have a significant effect on virus replication. On the other hand, the 3′ 98 nucleotides of the HCV genome were previously shown to be identical among other strains of genotypes 1a and 1b (Kolykhalov *et al.*, 1996; Tanaka *et al.*, 1996). Thus, use of the cassette vector would not alter this region except for addition of 3 nucleotides found in strain H77 between the poly(UC) region and the 3′ 98 conserved nucleotides. This study showed for the first time that it is possible to make infectious viruses containing terminal sequences specific for two different subtypes of the same major genotype of HCV.

The viremic pattern found in the early phase of the infection with the recombinant HC-J4 virus was similar to that observed for the recombinant H77 virus in chimpanzees (Bukh *et al.*, unpublished data; Kolykhalov *et al.*, 1997; Yanagi *et al.*, 1997). Two chimpanzees infected with the recombinant H77 virus developed hepatitis (Bukh *et al.*, unpublished data), whereas the chimpanzee infected with the recombinant HC-J4 virus in the present study had no biochemical evidence of hepatitis. It is well known that biological variation exists and some chimpanzees inoculated with HCV either lack or develop minimal evidence of hepatitis even though the same inoculum caused disease in other animals. Thus, the phenotype of the infectious clone of strain HC-J4 can be determined only by transfection of several chimpanzees.

The chimpanzee transfected in the present study was chronically infected with hepatitis G virus (HGV/GBV-C) (Bukh *et al.*, 1998) and had a titer of 10^6 GE/ml at the time of HCV transfection. Although HGV/GBV-C was originally believed to be a hepatitis virus, it does not cause hepatitis in chimpanzees (Bukh *et al.*, 1998) and may not replicate in the liver (Laskus *et al.*, 1997). Our study demonstrated that an ongoing infection of HGV/GBV-C did not prevent acute HCV infection in the chimpanzee model.

In two previous studies it was reported that RNA transcripts from cDNA clones of HCV-1 (genotype 1a) and HCV-N (genotype 1b), respectively, resulted in viral replication after transfection into human hepatoma cell lines (Dash *et al.*, 1997; Yoo *et al.*, 1995). In both of these studies, infectivity was reported for clones that did not contain the terminal 98 conserved nucleotides at the very 3′ end of the 3′ UTR. The viability of these clones was not tested *in vivo* and concerns were raised about the infectivity of these cDNA clones *in vitro* (Fausto, 1997). With the chimpanzee transfection system we have developed, we have begun to test directly the requirement for this region in a biologically relevant way.

Since the nearly complete ORF was amplified in long RT–PCR and cloned in one step, we were able to study the quasispecies nature of the cloning source as reflected by colinear mutations throughout the HCV genome. Our analysis showed that the quasispecies of HC-J4 found in this acute-phase pool represented at least three viral species and that a quasispecies in the HVR1 region of HCV paralleled quasispecies at multiple other positions throughout the genome. It was previously demonstrated that each chimpanzee inoculated with an acute-phase plasma pool of patient H (H77) became infected with a different dominant sequence (Farci *et al.*,

1994), indicating that multiple components of a quasispecies were infectious. Our findings in the present study indicate that simultaneous transmission of multiple species to a single chimpanzee occurred. The quasispecies of HC-J4 found in the acute-phase pool represented three of the four major quasispecies found in the inoculum (HC-J4/91, Okamoto *et al.*, 1992b). Our observation clearly illustrates the difficulties in accurately determining the evolution of HCV over time since multiple species with significant changes throughout the HCV genome can be present from the onset of the infection. Infection of chimpanzees with monoclonal viruses derived from the infectious clones should make it possible to perform more detailed studies of the evolution of HCV *in vivo* and its importance for viral persistence and pathogenesis.

In conclusion, we have constructed an infectious clone representing a genotype 1b strain of HCV. Our study demonstrated that it was possible to obtain an infectious clone of a second strain of HCV, that a consensus amino acid sequence was not absolutely required for infectivity, and that chimeras between the UTRs of two different genotypes could be viable. The availability of an infectious clone of a second strain of HCV, representing the most prevalent genotype in the world, should be important for further studies of viral replication and pathogenesis of HCV and should permit more detailed studies of the function of HCV proteins.

MATERIALS AND METHODS

Source of HCV genotype 1b

An infectious plasma pool (second chimpanzee passage) containing strain HC-J4, genotype 1b, was prepared from acute phase plasma of a chimpanzee experimentally infected with serum containing HC-J4/91 (Okamoto *et al.*, 1992b). The HC-J4/91 sample was obtained from a first chimpanzee passage during the chronic phase of hepatitis C about 8 years after experimental infection. The consensus sequence of the entire genome, except for the very 3′ end, was determined previously for HC-J4/91 (Okamoto *et al.*, 1992b).

Preparation of HCV RNA

Viral RNA was extracted from 100-μl aliquots of the HC-J4 plasma pool with the TRIzol system (GIBCO BRL). The RNA pellets were each resuspended in 10 μl of 10 mM dithiothreitol (DTT) with 5% (vol/vol) RNasin (20–40 units/μl) (Promega) and stored at −80°C or immediately used for cDNA synthesis.

Amplification and cloning of the 3′ UTR

A region spanning from NS5B to the conserved region of the 3′ UTR was amplified in nested RT–PCR (Yanagi *et al.*, 1997). The RNA was denatured at 65°C for 2 min, and cDNA was synthesized at 42°C for 1 h with Superscript II reverse transcriptase (GIBCO BRL) and primer H3′X58R (Yanagi *et al.*, 1997) in a 20-μl reaction volume. The cDNA mixture was treated with RNase H and RNase T1 (GIBCO BRL) at 37°C for 20 min. The first round of PCR was performed on 2 μl of the final cDNA mixture in a total volume of 50 μl with the Advantage cDNA polymerase mix (Clontech) and external primers H9261F and H3′X58R (Yanagi *et al.*, 1997). In the second round of PCR [internal primers H9282F and H3′X45R (Yanagi *et al.*, 1997)], 5 μl of the first round PCR mixture was added to 45 μl of the PCR reaction mixture. Each round of PCR (35 cycles) was performed in a DNA thermal cycler 480 (Perkin–Elmer) and consisted of denaturation at 94°C for 1 min (first cycle: 1 min 30 s), annealing at 60°C for 1 min, and elongation at 68°C for 2 min. After purification with QIAquick PCR purification kit (QIAGEN), digestion with *Hin*dIII and *Xba*I (Promega), and phenol/chloroform extraction, the amplified products were cloned into pGEM-9zf(−) (Promega) (Yanagi *et al.*, 1997).

Amplification and cloning of the entire ORF

A region from within the 5′ UTR to the variable region of the 3′ UTR of strain HC-J4 was amplified by long RT–PCR (Fig. 1) (Yanagi *et al.*, 1997). The cDNA was synthesized at 42°C for 1 h in a 20-μl reaction volume with Superscript II reverse transcriptase and primer J4-9405R (5′-GCCTATTGGCCTGGAGTGGTTAGCTC-3′) and treated with RNases as above. The cDNA mixture (2 μl) was amplified by long PCR with the Advantage cDNA polymerase mix and primers A1 (Bukh *et al.*, 1992; Yanagi *et al.*, 1997) and J4-9398R (5′-*AGGATGGC*CTTAAGGCCT-GGAGTGGTTAGCTCCCCGTTCA-3′). Primer J4-9398R contained extra bases (*italics*) and an artificial *Afl*II cleavage site (underlined). A single PCR round was performed in a Robocycler thermal cycler (Stratagene) and consisted of denaturation at 99°C for 35 s, annealing at 67°C for 30 s, and elongation at 68°C for 10 min during the first 5 cycles, 11 min during the next 10 cycles, 12 min during the following 10 cycles, and 13 min during the last 10 cycles.

After we digested the long PCR products obtained from strain HC-J4 with *Pin*AI (isoschizomer of *Age*I) and *Bfr*I (isoschizomer of *Afl*II) (Boehringer Mannheim), we attempted to clone them directly into a cassette vector (pCV), which contained the 5′ and 3′ termini of strain H77 (Yanagi *et al.*, 1997). However, we did not obtain any full-length clones. To improve the efficiency of cloning, we further digested the PCR product with *Bgl*II (Boehringer Mannheim) and cloned the two resultant genome fragments (L fragment: *Pin*AI/*Bgl*II, nt 156–8935; S fragment: *Bgl*II/*Bfr*I, nt 8936–9398) separately into pCV (Fig. 2). DH5α-competent cells (GIBCO BRL) were transformed and selected on LB agar plates containing 100 μg/ml ampicillin (SIGMA) and amplified in LB liquid cultures at 30°C for 18–20 h. Sequence analysis of nine

plasmids containing the S fragment (miniprep samples) and nine plasmids containing the L fragment (maxiprep samples) were performed as described previously (Yanagi *et al.*, 1997).

Three L fragments, each encoding a distinct polyprotein, were cloned into pCV-J4S9, which contained an S fragment encoding the consensus amino acid sequence of HC-J4, to construct three chimeric full-length HCV cDNAs (pCV-J4L2S, pCV-J4L4S, and pCV-J4L6S) (Fig. 2). Large-scale preparation of each clone was performed as described previously (Yanagi *et al.*, 1997) and the authenticity of each clone was confirmed by sequence analysis.

Sequence analysis

Both strands of DNA were sequenced with the ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit using *Taq* DNA polymerase (Perkin–Elmer) and about 90 specific sense and antisense primers. Analyses of genomic sequences, including multiple sequence alignments and tree analyses, were performed with GeneWorks (Oxford Molecular Group) (Bukh *et al.*, 1995).

We determined the consensus sequence of strain HC-J4 by direct sequencing of PCR products (nt 11–9412) and by sequence analysis of multiple cloned L and S fragments (nt 156–9371). The consensus sequence of the 3′ UTR (the 3′ variable region, the polypyrimidine tract, and the first 16 nt of the conserved region) was determined by analysis of 24 cDNA clones.

Intrahepatic transfection of a chimpanzee with transcribed RNA

Two *in vitro* transcription reactions were performed with each of the three full-length clones. In each reaction 10 μg of plasmid DNA linearized with *Xba*I (Promega) was transcribed in a 100-μl reaction volume with T7 RNA polymerase (Promega) at 37°C for 2 h as described previously (Yanagi *et al.*, 1997). Five microliters of the final reaction mixture was analyzed by agarose gel electrophoresis and ethidium bromide staining (Fig. 1). Each transcription mixture was diluted with 400 μl of ice-cold phosphate-buffered saline without calcium or magnesium and then the two aliquots from the same cDNA clone were combined, immediately frozen on dry ice, and stored at −80°C. Within 24 h after freezing the transcription mixtures were injected into the chimpanzee by percutaneous intrahepatic injection that was guided by ultrasound. Each inoculum was individually injected (five or six sites) into a separate area of the liver to prevent complementation or recombination. The chimpanzee was maintained under conditions that met all requirements for its use in an approved facility.

Serum samples were collected weekly from the chimpanzee and monitored for liver enzyme levels [alanine aminotransferase (ALT), gammaglutamyltranspeptidase (GGT), and isocitrate dehydrogenase (ICD)] and anti-HCV antibodies [second-generation ELISA test (Abbott)]. Weekly samples of 100 μl of serum were tested for HCV RNA in a sensitive nested RT–PCR assay (Bukh *et al.*, 1992; Yanagi *et al.*, 1996) with AmpliTaq Gold DNA polymerase. The genome equivalent (GE) titer of HCV was determined by testing 10-fold serial dilutions of the extracted RNA in the RT–PCR assay (Yanagi *et al.*, 1996). We defined one GE as the number of HCV genomes present in the highest dilution positive in the RT-nested PCR assay. Duplicate titers determined by the "Amplicor HCV Monitor Test" (Roche Diagnostic Systems) (data not shown) were equivalent to these except at weeks 2, 17, and 18 p.i., where the titers were below the detection limit of the Amplicor HCV Monitor Test.

To identify which of the three clones was infectious *in vivo* we amplified the NS3 region (nt 3659–4110) from the chimpanzee serum in a highly sensitive and specific nested RT–PCR assay with AmpliTaq Gold DNA polymerase and cloned the PCR products with a TA cloning kit (Invitrogen). In addition, the consensus sequence of the nearly complete genome (nt 11–9441) was determined by direct sequencing of overlapping PCR products.

ACKNOWLEDGMENTS

We thank Ms. L. Rasmussen and other staff members at *SAIC* (Frederick, MD) for assistance in sequence analysis, staff members at Bioqual (Rockville, MD) for providing animal care, and Mr. R. Engle at Georgetown University (Rockville, MD) for performing the second-generation ELISA and Amplicor HCV Monitor tests. We also thank Mr. C. L. Apgar for technical assistance. We are grateful to Drs. M. Mayumi, S. Mishiro, and H. Okamoto for providing plasma containing strain HC-J4/91 of HCV. This study was supported in part by NIH Contracts N01-AI-52705, N01-AI-45180, and N01-CO-56000.

Note added in proof. Following submission of this manuscript we found that chimpanzee 1500 was again positive for HCV RNA during weeks 21–24 p.i. (viral titer < 10^3 GE/ml). Thus we cannot determine at this time whether the infection with the chimeric recombinant 1b virus is chronic.

REFERENCES

Alter, M. J. (1997). Epidemiology of hepatitis C. *Hepatology* 26, 62S–65S.

Blight, K. J., and Rice, C. M. (1997). Secondary structure determination of the conserved 98-base sequence at the 3′ terminus of hepatitis C virus genome RNA. *J. Virol.* 71, 7345–7352.

Brechot, C. (1997). Hepatitis C virus 1b, cirrhosis, and hepatocellular carcinoma. *Hepatology* 25, 772–774.

Bukh, J., Purcell, R. H., and Miller, R. H. (1992). Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay. *Proc. Natl. Acad. Sci. USA* 89, 187–191.

Bukh, J., Purcell, R. H., and Miller, R. H. (1993). At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. *Proc. Natl. Acad. Sci. USA* 90, 8234–8238.

Bukh, J., Miller, R. H., and Purcell, R. H. (1995). Genetic heterogeneity of hepatitis C virus: Quasispecies and genotypes. *Semin. Liver Dis.* 15, 41–63.

Bukh, J., Emerson, S. U., and Purcell, R. H. (1997). Genetic heterogeneity of hepatitis C virus and related viruses. *In* "Viral Hepatitis and Liver Disease, Proceedings of IX Triennial International Symposium on Viral Hepatitis and Liver Disease, Rome, Italy, 1996" (M. Rizzetto, R. H.

Purcell, J. L. Gerin, and G. Verme, Eds.), pp. 167–175. Edizioni Minerva Medica, Turin.

Bukh, J., Kim, J. P., Govindarajan, S., Apgar, C. L., Foung, S. K. H., Wages, J., Yun, A. J., Shapiro, M., Emerson, S. U., and Purcell, R. H. (1998). Experimental infection of chimpanzees with hepatitis G virus and genetic analysis of the virus. *J. Infect. Dis.* 177, 855–862.

Choo, Q.-L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, A., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G., and Houghton, M. (1991). Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA* 88, 2451–2455.

Dash, S., Halim, A.-B., Tsuji, H., Hiramatsu, N., and Gerber, M. A. (1997). Transfection of HepG2 cells with infectious hepatitis C virus genome. *Am. J. Pathol.* 151, 363–373.

Farci, P., and Purcell, R. H. (1993). Natural history and experimental models. *In* "Viral Hepatitis: Scientific Basis and Clinical Management" (A. J. Zuckerman and H. C. Thomas, Eds.), pp. 241–267. Churchill Livingstone, Edinburgh.

Farci, P., Alter, H. J., Wong, D. C., Miller, R. H., Govindarajan, S., Engle, R., Shapiro, M., and Purcell, R. H. (1994). Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated *in vitro* neutralization. *Proc. Natl. Acad. Sci. USA* 91, 7792–7796.

Farci, P., Shimoda, A., Wong, D., Cabezon, T., De Gioannis, D., Strazzera, A., Shimizu, Y., Shapiro, M., Alter, H. J., and Purcell, R. H. (1996). Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. *Proc. Natl. Acad. Sci. USA* 93, 15394–15399.

Farci, P., Bukh, J., and Purcell, R. H. (1997). The quasispecies of hepatitis C virus and the host immune response. *Springer Semin. Immunopathol.* 19, 5–26.

Fausto, N. (1997). Replication of hepatitis C virus in tissue culture: A potential breakthrough in need of confirmation. *Am. J. Pathol.* 151, 361.

Fried, M. W., and Hoofnagle, J. H. (1995). Therapy of hepatitis C. *Semin. Liver Dis.* 15, 82–91.

Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., and Shimotohno, K. (1991). Hypervariable regions in the putative glycoprotein of hepatitis C virus. *Biochem. Biophys. Res. Commun.* 175, 220–228.

Honda, M., Brown, E. A., and Lemon, S. M. (1996). Stability of a stem-loop involving the initiator AUG controls the efficiency of internal initiation of translation on hepatitis C virus RNA. *RNA* 2, 955–968.

Hoofnagle, J. H. (1997). Hepatitis C: The clinical spectrum of disease. *Hepatology* 26, 15S–20S.

Houghton, M. (1996). Hepatitis C viruses. *In* "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley *et al.*, Eds.), 3rd ed. Lippincott-Raven, Philadelphia.

Ito, T., and Lai, M. M. C. (1997). Determination of the secondary structure of and cellular protein binding to the 3′-untranslated region of the hepatitis C virus RNA genome. *J. Virol.* 71, 8698–8706.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., and Shimotohno, K. (1990). Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc. Natl. Acad. Sci. USA* 87, 9524–9528.

Kolykhalov, A. A., Feinstone, S. M., and Rice, C. M. (1996). Identification of a highly conserved sequence element at the 3′ terminus of hepatitis C virus genome RNA. *J. Virol.* 70, 3363–3371.

Kolykhalov, A. A., Agapov, E. V., Blight, K. J., Mihalik, K., Feinstone, S. M., and Rice, C. M. (1997). Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* 277, 570–574.

Laskus, T., Radkowski, M., Wang, L.-F., Vargas, H., and Rakela, J. (1997). Lack of evidence for hepatitis G virus replication in the livers of patients coinfected with hepatitis C and G viruses. *J. Virol.* 71, 7804–7806.

Okamoto, H., Kurai, K., Okada, S., Yamamoto, K., Iizuka, H., Tanaka, T., Fukuda, S., Tsuda, F., and Mishiro, S. (1992a). Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: Comparative study of four distinct genotypes. *Virology* 188, 331–341.

Okamoto, H., Kojima, M., Okada, S., Yoshizawa, H., Iizuka, H., Tanaka, T., Muchmore, E. E., Peterson, D. A., Ito, Y., and Mishiro, S. (1992b). Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: Variability and stability. *Virology* 190, 894–899.

Rice, C. M. (1996). *Flaviviridae*: The viruses and their replication. *In* "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley *et al.*, Eds.), 3rd ed., Lippincott-Raven, Philadelphia.

Shimizu, Y. K., Iwamoto, A., Hijikata, M., Purcell, R. H., and Yoshikura, H. (1992). Evidence for *in vitro* replication of hepatitis C virus genome in a human T-cell line. *Proc. Natl. Acad. Sci. USA* 89, 5477–5481.

Shimizu, Y. K., Igarashi, H., Kiyohara, T., Cabezon, T., Farci, P., Purcell, R. H., and Yoshikura, H. (1996). A hyperimmune serum against a synthetic peptide corresponding to the hypervariable region 1 of hepatitis C virus can prevent viral infection in cell cultures. *Virology* 223, 409–412.

Simmonds, P., Holmes, E. C., Cha, T.-A., Chan, S.-W., McOmish, F., Irvine, B., Beall, E., Yap, P. L., Kolberg, J., and Urdea, M. S. (1993). Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J. Gen. Virol.* 74, 2391–2399.

Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I., and Okayama, H. (1991). Structure and organization of the hepatitis C virus genome isolated from human carriers. *J. Virol.* 65, 1105–1113.

Tanaka, T., Kato, N., Cho, M.-J., and Shimotohno, K. (1995). A novel sequence found at the 3′ terminus of hepatitis C virus genome. *Biochem. Biophys. Res. Commun.* 215, 744–749.

Tanaka, T., Kato, N., Cho, M.-J., Sugiyama, K., and Shimotohno, K. (1996). Structure of the 3′ terminus of the hepatitis C virus genome. *J. Virol.* 70, 3307–3312.

Tsuchihara, K., Tanaka, T., Hijikata, M., Kuge, S., Toyoda, H., Nomoto, A., Yamamoto, N., and Shimotohno, K. (1997). Specific interaction of polypyrimidine tract-binding protein with the extreme 3′-terminal structure of the hepatitis C virus genome, the 3′X. *J. Virol.* 71, 6720–6726.

Tsukiyama-Kohara, K., Iizuka, N., Kohara, M., and Nomoto, A. (1992). Internal ribosome entry site within hepatitis C virus RNA. *J. Virol.* 66, 1476–1483.

Weiner, A. J., Brauer, M. J., Rosenblatt, J., Richman, K. H., Tung, J., Crawford, K., Bonino, F., Saracco, G., Choo, Q.-L., Houghton, M., and Han, J. H. (1991). Variable and hypervariable domains are found in the regions of HCV corresponding to the Flavivirus envelope and NS1 proteins and the Pestivirus envelope glycoproteins. *Virology* 180, 842–848.

Weiner, A. J., Geysen, H. M., Christopherson, C., Hall, J. E., Mason, T. J., Saracco, G., Bonino, F., Crawford, K., Marion, C. D., Crawford, K. A., Brunetto, M., Barr, P. J., Miyamura, T., McHutchinson, J., and Houghton, M. (1992). Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: Potential role in chronic HCV infections. *Proc. Natl. Acad. Sci. USA* 89, 3468–3472.

Yamada, N., Tanihara, K., Takada, A., Yorihuzi, T., Tsutsumi, M., Shimomura, H., Tsuji, T., and Date, T. (1996). Genetic organization and diversity of the 3′ noncoding region of the hepatitis C virus genome. *Virology* 223, 255–261.

Yanagi, M., Bukh, J., Emerson, S. U., and Purcell, R. H. (1996). Contamination of commercially available fetal bovine sera with bovine viral diarrhea virus genomes: Implications for the study of hepatitis C virus in cell cultures. *J. Infect. Dis.* 174, 1324–1327.

Yanagi, M., Purcell, R. H., Emerson, S. U., and Bukh, J. (1997). Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc. Natl. Acad. Sci. USA* 94, 8738–8743.

Yoo, B. J., Selby, M. J., Choe, J., Suh, B. S., Choi, S. H., Joh, J. S., Nuovo, G. J., Lee, H.-S., Houghton, M., and Han, J. H. (1995). Transfection of a differentiated human hepatoma cell line (Huh7) with in vitro-transcribed hepatitis C virus (HCV) RNA and establishment of a long-term culture persistently infected with HCV. *J. Virol.* 69, 32–38.